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Adaptive Evolution of *Escherichia coli* Inactivated in the Phosphotransferase System Operon Improves Co-utilization of Xylose and Glucose Under Anaerobic Conditions

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Abstract Modification of the phosphoenolpyruvate/sugar phosphotransferase system (PTS) has shown improvement in sugar coassimilation in *Escherichia coli* production strains. However, in preliminary experiments under anaerobic conditions, *E. coli* strains with an inactive PTS and carrying pLOI1594, which encodes pyruvate decarboxylase and alcohol dehydrogenase from *Zymomonas mobilis*, were unable to grow. These PTS^- strains were previously evolved under aerobic conditions to grow rapidly in glucose (PTS^- $Glucose^+$ phenotype). Thus, in this work, applying a continuous culture strategy under anaerobic conditions, we generate a new set of evolved PTS^- $Glucose^+$ mutants, VH30N1 to VH30N6. Contrary to aerobically evolved mutants, strains VH30N2 and VH30N4 carrying pLOI1594 grew in anaerobiosis; also, their growth capacity was restored in a 100%, showing specific growth rates ($\mu \sim 0.12\ h^{-1}$) similar to the PTS^+ parental strain ($\mu = 0.11\ h^{-1}$). In cultures of VH30N2/pLOI1594 and VH30N4/pLOI1594 using a glucose–xylose mixture, xylose was totally consumed and consumption of sugars occurred in a simultaneous manner indicating that catabolic repression is alleviated in these strains. Also, the efficient sugar coassimilation by the evolved strains caused an increment in the ethanol yields.

Keywords Anaerobiosis · Evolved strains · PTS · Sugar co-utilization · Xylose

Introduction

Metabolic pathway engineering for the production of industrial chemicals, including biofuels using renewable biomass as raw material for carbon source, offers an

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alternative to the use of fossil fuels [1, 2]. However, the large-scale application of this approach requires microbes capable of fermenting glucose and xylose efficiently [3]. *Escherichia coli* offers several advantages as a biocatalyst for the production of a wide range of metabolites of commercial interest, including the capacity to grow in simple media, prior industrial use, and the ability to ferment a wide spectrum of sugars derived from lignocellulosic hydrolysates [4]. However, this bacteria displays catabolite repression, the ability to select from a mixture of carbon sources the one that affords the highest growth rate [5, 6]. This phenomenon is in part controlled by the phosphoenolpyruvate/sugar phosphotransferase system (PTS) [7, 8]. *E. coli* strains with inactivated PTS enzyme I, HPr, and IIA^{GLC} (PTS^- $Glucose^-$ phenotype) have been evolved using an aerobic continuous culture selection process, to generate strains displaying a PTS^- $Glucose^+$ phenotype. These strains displayed a specific growth rate (μ=0.4 h^{-1}) similar to that of the wild-type (μ=0.7 h^{-1}) when growing on glucose as the only carbon source under aerobic conditions [9]. It has been determined that the PTS^- $Glucose^+$ phenotype depends on the elevated level of the galactose proton simporter (*galP*) and the up-regulation of the glycolytic pathway [9–11]. In contrast to an isogenic wild-type strain, a PTS^- $Glucose^+$ strain has the capacity to simultaneously consume glucose and other carbon sources like arabinose, but glucose still exerted a partial repressive effect on xylose consumption [11–13]. Furthermore, in comparison with the wild-type strain W3110, the high-level expression of *galP* gene in *E. coli* VH32/pCLvGalP1 (W3110 PTS^-) restored the specific growth rate in 89% [14]. Also, a twofold increase in specific ethanol productivity was observed in the strain VH32/pCLvGalP1, when it has a plasmid that express pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adh*II) from *Zymomonas mobilis* [14].

In spite of the favorable characteristics of the PTS^- $Glucose^+$ mutants generated and characterized so far, it has been determined that they display defective growth under conditions of oxygen limitation (see "Results"). Therefore, further research is required to establish methods for generating PTS^- $Glucose^+$ strains suitable to be used in anaerobic processes.

In this work, we developed a continuous culture method for selecting *E. coli* W3110 PTS^- strains better adapted to grow under anaerobic conditions. Using this anaerobic strategy, an *E. coli* W3110 PTS^- $Glucose^-$ strain was evolved to obtain strains in which the $Glucose^+$ phenotype was reestablished. In these evolved PTS^- $Glucose^+$ strains, the specific growth rate was restored 100% of that observed in the PTS^+ wild-type parent strain. In experiments carried out using a glucose–xylose mixture, anaerobically evolved mutants displayed the ability to coassimilate both sugars. Also, the ethanol production was improved using the anaerobic evolved PTS^- $Glucose^+$ strains.

Materials and Methods

Bacterial Strains, Plasmids, and Media

E. coli strains genotype and plasmids used in this work are shown in Table 1. Strain W3110 is referred to as wild type. Cultures were grown in M9 mineral medium [15], pH adjusted to 7.0 with KOH 2 N, containing per liter: Na_2HPO_4 6 g, KH_2PO_4 3 g, NH_4Cl 1 g, and NaCl 0.5 g. The following components were sterilized separately, by filtration, and then added (per liter of final medium): 1 mL of $MgSO_4 \cdot 7H_2O$ 1 M, 1 mL of $CaCl_2$ 0.1 M, and 1 mL of thiamin/HCl 1 mg/mL.

Table 1 *E. coli* strains and plasmids used in this work

	Relevant genotype	Reference
Strains		
PB103	F^- Δ*lac*U169*trp*R, *tna*A2; it is a Trp^+ derivative of strain C534	[30]
NF9	PTS^- $Glucose^+$ derivative of PB103, selected by an aerobic continuous culture method	[9]
W3110	F^- λ^- INV(*rrn*D-*rrn*E)1	[16]
VH30	W3110 Δ*ptsH, ptsI, crr*::km^R, $Glucose^-$	[14]
VH32	VH30 Δ*lac*I, *lac*Z::*lox*P	[14]
VH30N1	PTS^- $Glucose^+$ derivative of VH30, selected by an anaerobic continuous culture method	This work
VH30N2	PTS^- $Glucose^+$ derivative of VH30, selected by an anaerobic continuous culture method	This work
VH30N4	PTS^- $Glucose^+$ derivative of VH30, selected by an anaerobic continuous culture method	This work
Plasmids		
pLOI1594	pUC19 derivative that harbors the *pdc* and *adhB* genes under the regulation region P_{zm4}	[17]
pCLv1GalP	pCL1920 derivative that harbors the *galP* fusion to the promoter *trc1*	[14]

Mutant Selection Under Anaerobic Conditions

With the purpose of generating PTS^- $Glucose^+$ mutants adapted to grow under anaerobiosis, VH30, a PTS^- $Glucose^-$ strain, was subjected to continuous culture under anaerobic conditions. VH30 strain was used to inoculate a 1-liter fermentor containing M9 medium supplemented with glucose 0.2% and kanamycin 33 μg/mL. Working conditions were 120 rpm, no aeration, 37 °C, and pH 7.0 (controlled by automatic additions of KOH 2 N). After the culture reached an optical density of 0.6 at 600 nm (OD_{600}), the continuous culture condition was initiated by feeding fresh M9–glucose–kanamycin medium at a dilution rate of 0.05 h^{-1} (before the feeding started fresh medium was sparged with nitrogen). After at least four residence times, the feed flow was increased to a dilution rate of 0.1 h^{-1}. This procedure was repeated using increases in the dilution rate of 0.05 h^{-1}, until the dilution rate reached a value of 0.4 h^{-1}. Before each increase in the dilution rate, samples were taken off from the chemostat. A 1:10 dilution of the samples was plated on MacConkey [15] –glucose 0.2%–kanamicin 33 μg/mL plates, and incubated 24 h at 37 °C. The rest of the sample was frozen for further analyses. The MacConkey media has a pH indicator dye that changes color in response to the acidic by-products of sugar metabolism. Colonies displaying homogeneous red color (PTS^- $Glucose^+$ phenotype) were selected for further studies.

Inoculum Preparation

Strains from frozen vials were inoculated in M9 mineral medium plates supplemented with tryptone 1%. This was supplemented with tryptone in order to improve the production of biomass. After incubation during 24 h at 30 °C, three fresh colonies were transferred into 250-mL flasks containing 50 mL M9-tryptone 1% media. Inocula were incubated for 12 h at

35 °C with 120 rpm in an orbital shaker (C24C incubator shaker; New Brunswick Scientific Inc., New Brunswick, NJ). Ampicillin (200 μg/mL) was added to all cultures of strains transformed with vector pLOI1594 [17].

Minifermentor Cultures

Cultures were carried out in 250-mL minifermentors (fleakers) [18] containing 200 mL of M9 medium supplemented with 10 g/L of glucose or xylose. For sugar mixture experiments, 5 g/L of glucose and 5 g/L of xylose were used. Working conditions were 100 rpm, no aeration, 35 °C, and pH 6 (controlled by automatic additions of KOH 2 N). All cultures were initiated an OD_{600} of 0.5. Cultures done with strains transformed with vector pLOI1594 were supplemented with ampicillin 200 μg/mL. All experiments were repeated (at least) in duplicate.

Analytical Methods

Biomass Concentration Determination

Biomass concentration was measured spectrophotometrically as OD_{600} (Lambda 11 Perkin Elmer, Pomona, CA) and converted to dry cellular weight using a standard curve (1 OD_{600} = 0.37 g/L of dry cellular weight). Culture data represent the average of at least two fermentations.

Sugar and Organic Acids Analysis

Glucose and xylose (refractive index detector), and acetic, formic, succinic, lactic and pyruvic acids (UV detector at 210 nm) were determined by high-performance liquid chromatography using an Aminex HPX-87H column (300×7.8 mm; Bio-Rad Laboratories, Hercules, CA) and H_2SO_4 5 mM as the mobile phase (0.5 mL/min) at 50 °C. Ethanol was analyzed by gas chromatography (6850 Series GC System Agilent, Wilmington, DE) using *n*-butanol as internal standard.

Results

Growth Kinetics for Wild-type and PTS^- $Glucose^+$ Strains Cultured Under Anaerobic Conditions

With the purpose of evaluating growth and ethanol production capacities of two PTS^- $Glucose^+$ strains under anaerobiosis, strains NF9 [9] and VH32/pCLvGalP1 [14] were cultured under limited oxygen conditions. Both NF9 and VH32/pCLvGalP1 are PTS^- $Glucose^+$ mutants; however, the $Glucose^+$ phenotype was restored by different approaches depending on the strain. In strain NF9, $Glucose^+$ phenotype was restored by adaptative evolution using an aerobic continuous culture [9]. In contrast, glucose import capacity in strain VH32 (PTS^- $Glucose^-$) was increased by transforming it with plasmid pCLvGalP1, which carries the *galP* gene transcribed by the strong promoter *trc*, displaying now a PTS^- $Glucose^+$ phenotype [14]. Both strains, NF9 and VH32/pCLvGalP1, were cultured on M9 mineral medium with glucose under anaerobic conditions. Mutants displayed the same growth rate as their parental wild-type strains, PB103 and W3110, respectively (data not

shown). However, when these four strains were transformed with the ethanologenic vector pLOI1594 and cultured in anaerobiosis, the PTS$^-$ Glucose$^+$ derivatives displayed highly reduced growth capacity (Fig. 1). Since PTS$^+$ strains (PB103 and W3110) carrying plasmid pLOI1594 were able to grow under anaerobiosis, the previous result suggests that PTS$^-$ strains cannot cope with the metabolic burden imposed by plasmid pLOI1594 under anaerobiosis. Thereby, to obtain robust PTS$^-$ Glucose$^+$ strains capable of growing and producing ethanol under anaerobic conditions, it was necessary to modify the original method reported by Flores et al. [9] for the generation and isolation of PTS$^-$ Glucose$^+$ strains (see "Material and methods").

Generation and Characterization of PTS$^-$ Glucose$^+$ Evolved Strains Under Anaerobic Conditions

In order to generate PTS$^-$ Glucose$^+$ strains better adapted to grow under anaerobic conditions, strain VH30 (PTS$^-$ Glucose$^-$) was subjected to continuous culture under an anaerobic environment. Following this protocol six mutants (one per each dilution rate stage used), VH30N1 to VH30N6 were isolated. These six mutants were characterized under anaerobic conditions using mineral medium with 10 g/L of glucose. Strains VH30N1 to VH30N6 displayed specific rates of growth and glucose consumption similar among them and to the parental W3110 strain (data not show). Therefore only three mutants, VH30N1, VH30N2, and VH30N4, were selected for further characterization of sugar coassimilation and ethanol production capacities. Table 2 shows a comparison of specific growth rates between these anaerobically PTS$^-$ Glucose$^+$ evolved mutants and one PTS$^-$ Glucose$^+$ mutant (NF9) isolated under aerobic conditions, cultured under anaerobiosis with glucose 10 g/L. Strains VH30N1, VH30N2, and VH30N4 showed specific growth rates 2.25, 2.75, and 3.25 times higher than that from NF9, respectively, indicating the better adaptation of new PTS$^-$ Glucose$^+$ evolved strains to grow under oxygen-limited conditions.

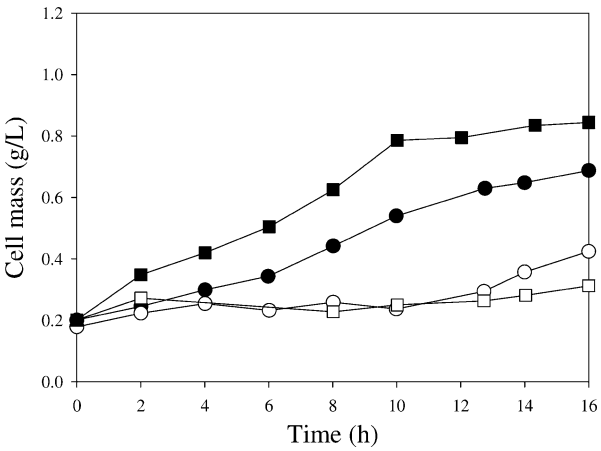

Fig. 1 Growth of *E. coli* wild-type (PB103 and W3110) and PTS$^-$ Glucose$^+$ mutants (NF9 and VH32/pCLvGalP1) strains transformed with pLOI1594 under anaerobic conditions. Cultures using mineral media supplemented with glucose 10 g/L. *Closed circles*, PB103/pLOI1594; *open circles*, NF9/pLOI1594; *closed squares*, W3110/pLOI1594; *open squares*, VH32/pCLvGalP1/pLOI1594

Table 2 Comparison of specific growth rates (μ) of *E. coli* wild-type strains and PTS$^-$ Glucose$^+$ mutants from cultures under anaerobic conditions

Wild-type strain	μ (h^{-1})	PTS$^-$ Glucose$^+$ strain	μ (h^{-1})
PB103	0.10 (0.01)	NF9[a]	0.04 (0.01)
W3110	0.11 (0.01)	VH30N1[b]	0.09 (0.01)
		VH30N2[b]	0.11 (0.00)
		VH30N4[b]	0.13 (0.02)

[a] NF9 mutant isolated under aerobic continuous culture selection

[b] VH30N*x* derivatives isolated under anaerobic continuous culture selection

Data in parenthesis indicate standard error. Data shown is from anaerobic cultures in mineral medium with glucose 10 g/L

Growth and Ethanol Production Profiles by PTS$^-$ Glucose$^+$ Evolved Mutants Using Glucose Under Anaerobic Conditions

Ethanologenic derivatives of VH30N1, VH30N2, and VH30N4 strains were generated by transforming them with plasmid pLOI1594. Using these strains, growth and production kinetics were determined in M9-mineral medium with 10 g/L glucose under anaerobiosis. In contrast with the results observed for strains NF9/pLOI1594 and VH32/pCLvGalP1/pLOI1594 (Fig. 1), VH30N2 and VH30N4 mutants transformed with pLOI1594 were able to grow under anaerobiosis (Fig. 2). However, strain VH30N1/pLOI1594 exhibited a low growth rate, and the maximum biomass reached was 0.3 g/L, three times lower than that observed for VH30N2/pLOI1594 and VH30N4/pLOI1594 (Fig. 2). Specific growth rates of strains VH30N2 and VH30N4 carrying pLOI1594 were 0.14 and 0.13 h^{-1}, respectively. These values were similar to that of W3110/pLOI1594 (μ=0.12 h^{-1}; Table 3). Thus, strains VH30N2 and VH30N4 were

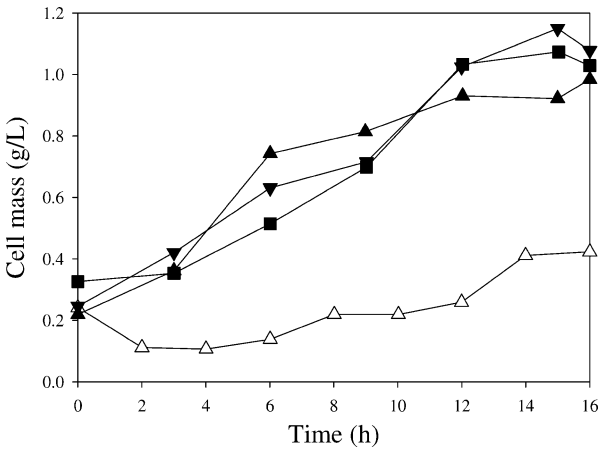

Fig. 2 Growth of *E. coli* wild-type strain (W3110) and PTS$^-$ Glucose$^+$ evolved strains (VH30N1, VH30N2 and VH30N4) transformed with pLOI1594 under anaerobic conditions. Cultures performed using mineral media supplemented with glucose 10 g/L. *Closed squares*, W3110/pLOI1594; *open triangles up*, VH30N1/pLOI1594; *closed triangles up*, VH30N2/pLOI1594; *closed triangles down*, VH30N4/pLOI1594

Table 3 Kinetic parameters of *E. coli* W3110 and PTS$^-$ Glucose$^+$ mutants transformed with plasmid pLOI1594 obtained from cultures under anaerobic conditions

Strain	μ, specific growth rate (h^{-1})	q_s, specific sugar consumption rate ($g_s/g_{biomass} \cdot h$)	q_p, specific ethanol production rate ($g_{EtOH}/g_{biomass} \cdot h$)	$Y_{EtOH/biomass}$, ethanol/biomass yield ($g_{EtOH}/g_{biomass}$)	$Y_{EtOH/sugar}$, ethanol/sugar yield (g_{EtOH}/g_s)
Glucose 10 g/L					
W3110/pLOI1594	0.12 (0.0)[a]	1.14 (0.19)	0.17 (0.03)	1.68 (0.00)	0.14 (0.00)
VH30N2/pLOI1594	0.14 (0.01)	1.26 (0.3)	0.17 (0.02)	1.43 (0.05)	0.12 (0.00)
VH30N4/pLOI1594	0.13 (0.02)	1.17 (0.13)	0.15 (0.01)	2.10 (0.01)	0.12 (0.00)
Xylose 10 g/L					
W3110/pLOI1594	0.11 (0.01)[a]	0.94 (0.10)	0.19 (0.02)	1.78 (0.00)	0.14 (0.02)
VH30N2/pLOI1594	0.09 (0.00)	0.86 (0.00)	0.20 (0.01)	2.35 (0.05)	0.20 (0.00)
VH30N4/pLOI1594	0.08 (0.00)	0.76 (0.06)	0.19 (0.00)	2.16 (0.14)	0.20 (0.02)
Glucose 5.0 g/L–xylose 5.0 g/L mixture					
W3110/pLOI1594	0.12 (0.00)[a] Glc 0 Xyl	1.01 (0.04) Glc 0.09 (0.03) Xyl	0.12 (0.00) Glc 0.04 (0.0) Xyl	2.1 (0.14)	0.19 (0.00)
VH30N2/pLOI1594	0.11 (0.00) Glc 0 Xyl	1.06 (0.02) Glc+Xyl 0.34 (0.01) Xyl	0.2 (0.05) Glc+Xyl 0.11(0.01) Xyl	3.48 (0.24)	0.24 (0.01)
VH30N4/pLOI1594	0.14 (0.00) Glc 0 Xyl	1.1 (0.05) Glc+Xyl 0.5 (0.00) Xyl	0.15 (0.01) Glc+Xyl 0.08 (0.01) Xyl	2.64 (0.18)	0.21 (0.00)

[a] Data in parenthesis indicate standard error

Data shown is from cultures grown in M9 mineral media supplemented with glucose or xylose and a mixture of both sugars as indicated

selected for further studies. The parameters calculated for cultures with these strains: specific sugar consumption rate (q_s), specific ethanol production rate (q_{EtOH}), ethanol/biomass yield ($Y_{EtOH/biomass}$), and ethanol/sugar yield ($Y_{EtOH/sugar}$) are summarized in Table 3.

No significant differences were found for q_s and q_{EtOH} when comparing anaerobic evolved mutants and W3110/pLOI1594 strain. Ethanol/biomass yield was 15% lower for VH30N2/pLOI1594 and 25% higher for VH30N4/pLOI1594, in comparison with the parental strain (Table 3). Finally, ethanol/sugar yield was 15% lower for both mutant strains. The organic acids/sugar yield ratio was similar for all strains (data not shown).

Growth and Ethanol Production Profiles by PTS$^-$ Glucose$^+$ Evolved Mutants Using Xylose Under Anaerobic Conditions

Xylose is the most abundant pentose derived from agricultural residues, and it is desirable that microorganisms ferment this sugar in a rapid and efficient manner to produce ethanol [3]. To evaluate if the anaerobic PTS$^-$ Glucose$^+$ evolved mutants were able to assimilate xylose, cultures using minimum medium with 10 g/L of this sugar under anaerobiosis were carried out. All strains grown in xylose showed lower values of μ compared with those observed for cultures using glucose as carbon source. VH30N2/pLOI1594 and VH30N4/pLOI1594 strains showed a μ of 0.09 and 0.08 h^{-1}, respectively (Table 3), 20% and 27% lower than the μ of W3110/pLOI1594 grown in xylose.

Regarding xylose consumption, no significant difference in q_s was observed among strains (Table 3). Ethanol production rate was similar for all strains; however, $Y_{EtOH/biomass}$ values were 32% and 21% higher for VH30N2/pLOI1594 and VH30N4/pLOI1594, and $Y_{EtOH/sugar}$ was 43% higher for both strains than the corresponding values for W3110/pLOI1594 (Table 3). For organic acid production, both mutants produced 50% less succinic acid than the wild type, and no significant difference was found for lactic, acetic, and formic acid production (data not shown).

Growth, Sugar Utilization, and Ethanol Production Profiles by PTS$^-$ Glucose$^+$ Evolved Mutants Using a Glucose–xylose Mixture Under Anaerobic Conditions

Pentoses, like xylose, are the major components from hemicellulosic hydrolysates [19]. In *E. coli* as an effect of catabolic repression xylose is not metabolized until glucose is completely consumed. Thus, to evaluate xylose-glucose cofermentation, cultures using a mixture of xylose 5.0 g/L and glucose 5.0 g/L were carried out. Evaluation was performed for strains W3110, VH30N2, and VH30N4, all carrying the pLOI1594 plasmid. For these experiments, two q_s and q_p values were calculated, one before and the other one after glucose depletion (Table 3).

The parental strain W3110 transformed with plasmid pLOI1594 showed a μ of 0.12 h^{-1}. This growth rate was the same than the observed for W3110/pLOI1594 using only glucose as carbon source (Table 3). However, when glucose from the mixture was exhausted, the growth of strain W3110/pLOI1594 stopped (Fig. 3a), reaching only 0.6 g/L as maximum cell mass, 40% lower than the obtained using glucose (10 g/L) as carbon source (Fig. 2). Subsequently to glucose depletion, the growth capacity of strain W3110/pLOI1594 could not be restored since the xylose consumption was slow and incomplete (Fig. 3b). The q_s of xylose observed was 0.09 $g_s/g_{biomass}{\cdot}h$, 90% lower than the xylose q_s from the cultures using only xylose (Table 3). These data suggest that 5 g/L of glucose caused a catabolic repression effect on xylose consumption in the cultures of strain W3110/pLOI1594. Also, incomplete consumption of xylose caused that strain W3110/pLOI1594 accumulated only 0.8 g/L of ethanol (Fig. 3c), the lowest amount observed for the three strains evaluated.

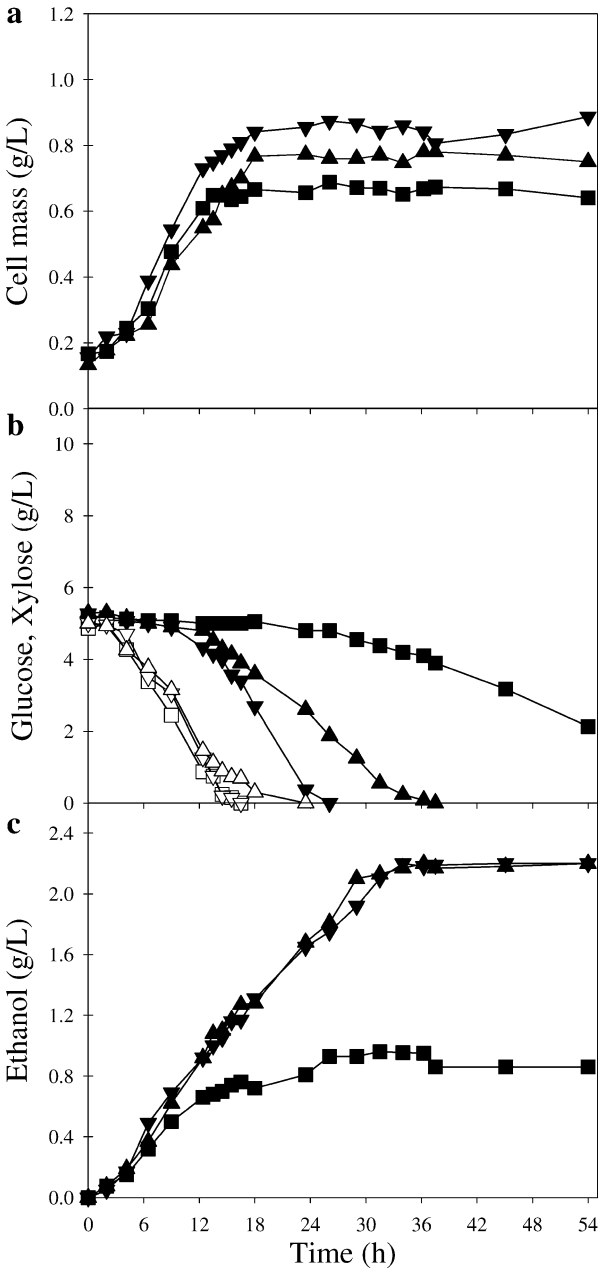

Fig. 3 Growth kinetics of *E. coli* wild-type strain (W3110) and PTS^{-} Glucose^{+} evolved strains (VH30N2 and VH30N4) transformed with pLOI1594 under anaerobic conditions. Cultures performed using mineral media supplemented with the simultaneous presence of glucose (5.0 g/L) and xylose (5.0 g/L). *Closed squares*, W3110/pLOI1594; *closed triangles up*, VH30N2/pLOI1594; *closed triangles down*, VH30N4/pLOI1594. **a** Growth. **b** Sugar consumption; glucose, *open symbols*, and xylose, *closed symbols*. **c** Ethanol production

Strains VH30N2/pLOI1594 and VH30N4/pLOI1594 grown in the glucose 5 g/L–xylose 5 g/L mixture reached a similar maximum final biomass of 0.8 g/L (Fig. 3a). However, strain VH30N2/pLOI1594 showed a μ of 0.11 h^{-1}, 21% lower in comparison with the μ value for VH30N4/pLOI1594, and also with the μ of VH30N2/pLOI1594 obtained when glucose was used as the only carbon source (Table 3). Regarding sugar consumption, in contrast to that observed for strain W3110/pLOI1594, evolved strains VH30N2/pLOI1594 and VH30N4/pLOI1594 were capable to consume xylose and glucose in a simultaneous manner (Fig. 3b). Xylose was totally consumed in 37 and 25 h of fermentation by VH30N2/pLOI1594 and VH30N4/pLOI1594, respectively. These results confirmed that catabolic repression in the anaerobically evolved strains VH30N2/pLOI1594 and VH30N4/pLOI1594 was alleviated. Strains VH30N2/pLOI1594 and VH30N4/pLOI1594 produced 2.1 g/L of ethanol as maximum (Fig. 3c), 2.6 times higher than that with W3110/pLOI1594 (Table 3). Also, q_p and $Y_{EtOH/biomass}$ values of VH30N2 and VH30N4 carrying pLOI1594 using glucose 5 g/L–xylose 5 g/L mixture were the highest obtained in comparison with all carbon sources evaluated. These higher values could be related to the efficient xylose–glucose coassimilation by these anaerobically evolved strains.

Discussion

The aim of the current work was to evaluate under anaerobic conditions the growth and sugar utilization capacities of *E. coli* W3110 PTS^- $Glucose^+$ mutants carrying the ethanologenic pLOI1594 plasmid. Strain VH32/pCLvGalP1 transformed with pLOI1594 plasmid has been shown to display good growth and ethanol production capacities under aerobic conditions [14]. Nevertheless, when we cultured this PTS^- $Glucose^+$ strain under anaerobic conditions, its growth was severely reduced. We also evaluated under anaerobic conditions the PTS^- $Glucose^+$ strain NF9 transformed with pLOI1594, which was obtained by adaptative evolution under aerobic conditions. A severe reduction in its growth capacity was also observed for this strain. These results suggest that the metabolic load imposed by pLOI1594 in aerobic adapted PTS^- $Glucose^+$ strains exceeds their capacity to grow under anaerobiosis. A similar effect was reported by Madhavan et al. [20], in which an aerobically improved *Saccharomyces cerevisiae* strain showed lower ethanol yields and production rates when was cultured under anaerobic conditions.

Considering the previous results, it was necessary to develop a method for obtaining PTS^- $Glucose^+$ strains better adapted to grow under anaerobic ethanol production conditions. No aeration, lower dilution rates, and a lower initial biomass were the adjustments performed to the adaptative evolution method described by Flores et al. [9]. This strategy allowed the isolation of PTS^- mutants that were evolved to restore the $Glucose^+$ phenotype that also displayed robustness under anaerobic conditions. The μ in these anaerobically evolved PTS^- $Glucose^+$ strains was restored to 100% in comparison with the parent PTS^+ stain.

Characterization of strains VH30N2/pLOI1594 and VH30N4/pLOI1594 using glucose, xylose, and a mixture of both sugars were conducted to determine if catabolic repression was still present in these anaerobically adapted PTS^- $Glucose^+$ strains. Cultures of VH30N2/pLOI1594 and VH30N4/pLOI1594 using glucose grew faster and exhibited the highest values of μ and q_s, compared with cultures using xylose. Nevertheless, ethanol yields for cultures with glucose were low. Cultures of VH30N2/pLOI1594 and VH30N4/pLOI1594 using xylose showed 45% and 49% lower values of μ compared with the observed using glucose, respectively. The low μ observed in these cultures with xylose under anaerobiosis is explained by the fact that anaerobic fermentation of xylose to pyruvate only yields 0.67 net

ATP per xylose (in comparison with 2 ATP per glucose), due to the ATP requirements for xylose transport and xylulose phosphorylation [21, 22]. Also a particular problem that is often encountered in fermentations of biomass hydrolysates is the slow and incomplete use of pentoses, like xylose when glucose is present, using only 75%–80% of the total xylose [2, 23, 24]. VH30N2/pLOI1594 and VH30N4/pLOI1594 evolved strains were able to assimilate all the xylose present in the culture media faster than the PTS^+ W3110 strain, even in the presence of glucose. In cultures using a sugar mixture, the consumption of glucose and xylose was simultaneous, evidencing that the catabolic repression was abolished in VH30N2/pLOI1594 and VH30N4/pLOI1594 evolved strains. Several approaches have focused to enhance xylose uptake from mixtures of glucose and xylose in *E. coli*, including replacement of the native cyclic AMP receptor protein (CRP) with a cyclic AMP-independent mutant (CRP*) [25]; overexpression of the native *E. coli* xylose transporters, the d-xylose/proton symporter XylE, and the d-xylose ABC transporter XylFGH [26]; a cofermentation strategy that uses two substrate-selective strains of *E. coli*, one that is unable to consume glucose but consumes only xylose and one that is unable to consume xylose but consumes only glucose, consuming both sugars more quickly when compared with an approach based on using a single organism to consume both sugars simultaneously [27]. In addition, modification of the PTS has shown improvement in the coassimilation of sugars in *E. coli ptsG*$^-$ mutants for the production of ethanol [24], l-lactic acid [23], succinic acid [28], and polyhydroxyalkanoates [29]. In comparison with the PTS^- mutants before mentioned, VH30N2/pLOI1594 and VH30N4/pLOI1594 evolved mutants have deleted the *ptsHI* and *crr* genes; thus, the main PEP consumption pathway is eliminated, increasing the availability of PEP in the cell, which can be channeled to biosynthesis or to metabolite production pathways [31]. Cultures with VH30N2/pLOI1594 and VH30N4/pLOI1594 evolved strains, using xylose or glucose–xylose mixtures, showed higher ethanol/biomass and ethanol/sugar yields in comparison with those obtained using glucose as carbon source.

In this work, a novel adaptative evolution method was applied to obtain robust PTS^- $Glucose^+$ strains suitable to be used as ethanol production strains under anaerobic conditions. Further characterization of these VH30N2 and VH30N4 evolved strains, including transcriptome and metabolome analyses, will help in defining metabolic engineering strategies for further ethanol productivity improvement. Also, these *omic*-studies will help to identify the responsible changes for the improved sugar coassimilation observed in VH30N2 and VH30N4 evolved strains, traits that could be transferable to other strains and extend the knowledge in this field. VH30N2 and VH30N4 evolved mutants, where the catabolic repression was mainly alleviated, are promising strains to ferment biomass hydrolysates for the production of fuels and commodity chemicals under anaerobic conditions. The utilization of evolved strains that can grow in specific metabolic conditions provides an important tool for increasing the production of relevant metabolites in industrial microorganisms.

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